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# Acetolactate Synthase Activity in Developing Maize (*Zea mays* L.) Kernels

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MICHAEL J. MUHITCH
*Seed Biosynthesis Research Unit, United States Department of Agriculture, Agricultural Research Service, Northern Regional Research Center, Peoria, Illinois 61604*

## ABSTRACT

**Acetolactate synthase (EC 4.1.3.18) activity was examined in maize (*Zea mays* L.) endosperm and embryos as a function of kernel development. When assayed using unpurified homogenates, embryo acetolactate synthase activity appeared less sensitive to inhibition by leucine + valine and by the imidazolinone herbicide imazapyr than endosperm acetolactate synthase activity. Evidence is presented to show that pyruvate decarboxylase contributes to apparent acetolactate synthase activity in crude embryo extracts and a modification of the acetolactate synthase assay is proposed to correct for the presence of pyruvate decarboxylase in unpurified plant homogenates. Endosperm acetolactate synthase activity increased rapidly during early kernel development, reaching a maximum of 3 micromoles acetoin per hour per endosperm at 25 days after pollination. In contrast, embryo activity was low in young kernels and steadily increased throughout development to a maximum activity of 0.24 micromole per hour per embryo by 45 days after pollination. The sensitivity of both endosperm and embryo acetolactate synthase activities to feedback inhibition by leucine + valine did not change during kernel development. The results are compared to those found for other enzymes of nitrogen metabolism and discussed with respect to the potential roles of the embryo and endosperm in providing amino acids for storage protein synthesis.**

Storage protein synthesis in the developing maize kernel begins around 10 d after pollination and creates a large demand for amino acids. Most of the nitrogen arriving via the translocation stream is in the form of aspartic acid and the amides asparagine and glutamine (2). In order to meet the demands of storage protein synthesis for the various amino acids, the nitrogen from a portion of these transport compounds must be incorporated into other amino acids within the developing kernel. The activities of several enzymes of amide metabolism and amino acid biosynthesis have been detected in maize endosperm. Studies with labeled amino acids also indicate that there is active nitrogen metabolism within the developing endosperm (17, 20). Two recent reports (3, 4) suggest that the embryo might also play a role in providing amino acids for endosperm storage synthesis in the later stages of kernel development.

Leucine is one of the most abundant amino acids in mature maize endosperm (21). Since leucine levels in the translocation stream leading to the ear are low (2), active *de novo* branched-chain amino acid synthesis would be expected to occur in developing maize kernels. ALS,[1] also known as acetohydroxyacid synthase, is the first enzyme in the common biosynthetic pathway leading to leucine, valine, and isoleucine. Much recent interest in this enzyme has resulted from its identification as the target of two classes of herbicides, the imidazolinones (15) and the sulfonylureas (13). In this report, the results of studies on ALS activity in developing maize kernels are presented. Evidence is presented which suggests that PDC contributes to the apparent ALS activity of crude embryo extracts. A modification of the ALS assay is proposed for measuring ALS activity in plant homogenates which contain PDC. ALS activities in maize endosperm and embryo homogenates were determined as a function of kernel development and these results are discussed within the context of the roles that the embryo and the endosperm play in providing amino acids for storage protein synthesis during kernel development.

## MATERIALS AND METHODS

**Plant Materials.** Hybrid maize (*Zea mays* L., W64A × A619) was grown under field conditions in the summer of 1985. Developing kernels were either used immediately for chromatography studies or frozen in liquid $N_2$, lyophilized, and stored at −80°C for developmental studies.

**Chemicals and Chromatographic Materials.**[2] α-Naphthol and acetoin were purchased from the Eastman Kodak Co. and the Aldrich Chemical Co., respectively. Imazapyr was obtained from American Cyanamid Co. All other chemicals were purchased from the Sigma Chemical Co. Mono Q anion exchange columns, PD-10 Sephadex G-25 columns, a Superose 6 gel filtration column, and other FPLC system components were purchased from Pharmacia Inc.

**Enzyme Isolation.** Maize kernel tissues were homogenized with a prechilled mortar and pestle using 50 mM K-phosphate (pH 7.4), containing 5 mM $MgCl_2$, 5 mM EDTA, 10% (v/v) glycerol, 5 mM pyruvate, 10 mM each of leucine and valine, 100 μM FAD, and 1 mM TPP. The isolation buffer is a modification of that described previously (15). The homogenate was filtered through four layers of cheesecloth and clarified by centrifugation at 20,000 *g* for 20 min. Ammonium sulfate was added to the supernatant (as a saturated solution) to 65% of saturation and ALS was pelleted by centrifugation as described above. For studies of concentrated homogenates, the proteins were resuspended in isolation buffer minus leucine, valine, and pyruvate and desalted using a PD-10 Sephadex G-25 column equilibrated in the same buffer. All operations were carried out at 4°C.

**Enzyme Assays.** The ALS assay of Miflin (9) was used with some modifications. The enzyme preparation was incubated in

[1] Abbreviations: ALS, acetolactate synthase (EC 4.1.3.18); PDC, pyruvate decarboxylase (EC 4.1.1.1); FPLC, Fast Protein Liquid Chromatography, a trademark of Pharmacia, Inc.; TPP, thiamine pyrophosphate; FAD, flavin adenine dinucleotide; DAP, days after pollination.

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that might also be suitable.

a 1 ml assay medium containing 50 mM potassium phosphate (pH 7.0), 40 mM sodium pyruvate, 10 mM $MgCl_2$, 0.62 mM TPP, and 34 $\mu$M FAD at 37°C. After 1 h, 0.2 ml of 30% (v/v) sulfuric acid was added to stop the reaction and to convert the acetolactate produced to acetoin. The quenched reaction samples were centrifuged at 1700 *g* for 10 min to remove protein and acetoin was quantitated using the color reaction described by Westerfield (22), as follows: 1 ml each of 0.5% (w/v) creatine and 1.0% (w/v) $\alpha$-naphthol in 4 N NaOH, was added to the supernatants, and the tubes were incubated at room temperature with periodic mixing. After 1 h, absorbance at 520 nm was measured. Color formation was linear with incubation time at 37°C for at least 60 min. The activity of control assay samples were subtracted from each sample. The control assays were either acidified prior to the addition of the enzyme (standard assay control) or 0.1 mM imazapyr was included in the assay buffer. The type of control assay used is indicated in the figure legends and table footnotes. ALS activities measured using the imazapyr-containing controls (*i.e.* Fig. 3) are expressed as $\mu$mol acetoin/h·tissue unit using a standard curve prepared with commercially available acetoin.

A coupled assay procedure was used to assay PDC in which the acetaldehyde produced by the enzyme was reduced to ethanol in the presence of NADH-dependent alcohol dehydrogenase (7).

**Chromatographic Separations.** Ion exchange chromatography was performed using a HR 10/10 mono Q anion exchange column and the FPLC system. Ammonium sulfate-precipitated protein pellets were resuspended in 2.5 ml of ion exchange starting buffer, consisting of 50 mM bis-tris (bis[2-hydroxyethyl]-imino-tris-[hydroxymethyl]-methane), (pH 6.5), 5 mM pyruvate, 1 mM EDTA, and 10% (v/v) glycerol and desalted using mini-columns of Sephadex G-25 which had been equilibrated in the same buffer. Portions of the desalted enzyme were applied to the ion exchange column. The column was then washed with 60 ml of starting buffer and the enzyme was eluted at a flow rate of 1.5 ml/min, with a 160 ml, 0 to 0.3 M KCl gradient, in starting buffer. Four-ml fractions were collected.

Gel filtration was performed with a Superose 6 column and the FPLC system. Ammonium sulfate-precipitated protein pellets were resuspended in 2.5 ml of ALS gel filtration medium (12), which consisted of 50 mM K-phosphate (pH 6.5) containing 1 mM EDTA, 10 $\mu$M FAD, 5 mM pyruvate, 15% (v/v) ethylene glycol, 100 mM KCl, and 10 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate). One-tenth ml samples were applied to the column, which was equilibrated in the same buffer, and ALS activities were eluted at a flow rate of 0.2 ml/min. One-ml fractions were collected. Coliphage $T_7$ DNA (approximate mol wt $25 \times 10^6$ D), was used to determine the void volume of the column.

**Miscellaneous Analytical Methods.** Total nitrogen was determined by a micro-Kjeldahl method (1). Conductivity was measured with a Yellow Springs Inc. conductance meter, model 32 (Yellow Springs, OH). A standard curve was made by measuring the conductance of KCl solutions prepared in ion exchange starting buffer.

## RESULTS AND DISCUSSION

**PDC Interference in ALS Assays of Crude Maize Embryo Extracts.** When maize endosperm extracts were assayed for ALS activity using the standard assay procedure, creatine and $\alpha$-naphthol dependent color formation was inhibited by including either the plant ALS feedback inhibitors leucine + valine (10) or the ALS inhibitor imazapyr (15) in the assay medium (Table I). In contrast, color development was less sensitive to inhibition by either leucine + valine or imazapyr when maize kernel embryos were assayed by this same procedure. These results could be explained by either (a) the presence of (an) embryo ALS isozyme(s) which are (is) insensitive to inhibition by leucine + valine and imazapyr or (b) the presence of another enzyme which contributes to apparent ALS activity in embryo extracts. The first explanation seems unlikely in that surveys of wide varieties of plants indicate that ALS activities are inhibited by leucine + valine (10) and by imazapyr (BK Singh, personal communication) at levels far below those used here. With respect to the second explanation, PDC, which catalyzes the formation of acetaldehyde and $CO_2$ from pyruvate, is likely to contribute to apparent ALS activity in embryo extracts under standard ALS assay conditions for the following reasons: PDC utilizes the same substrate (pyruvate) and two of the same cofactors ($Mg^{2+}$ and TPP) as ALS. Also, acetaldehyde, a product of PDC activity, forms a colored complex in the presence of creatine and $\alpha$-naphthol (16) and therefore would contribute to apparent ALS activity. Third, higher plant and yeast PDCs catalyze a second reaction when assayed *in vitro* in which 1 mol of acetaldehyde is condensed with a second resulting in the formation of $CO_2$ and acetoin (6). The acetoin produced as a result of PDC activity would contribute to apparent ALS activity under standard assay conditions. Finally, maize kernels are a rich source of PDC activity (7) and, within the kernel, this activity is localized in the embryo (TC Lee, personal communication). In order to determine if the relative insensitivity of embryo extracts to inhibition by leucine + valine and imazapyr in the ALS assay was due to interfering PDC, embryo extracts were subjected to gel filtration and anion exchange chromatographies to physically separate ALS from PDC.

Table I. *Creatine and $\alpha$-Naphthol Dependent Color Formation of Maize Endosperm and Embryo Homogenates Incubated Under Acetolactate Synthase Assay Conditions*

Homogenates of 25 of 40 DAP embryos and 6 of 20 DAP endosperms were prepared as described in "Materials and Methods." Acetolactate synthase assays were performed using 50 $\mu$l (embryo) or 25 $\mu$l (endosperm) of the enzyme preparations incubated at 37°C for 1 h.

| | Embryo | Endosperm |
|---|---|---|
| | *absorbance at 520 nm* | |
| Assay conditions[a] | | |
| No additions | 0.593 | 0.755 |
| +10 mM leucine + valine[b] | 0.255 (43%)[c] | 0.143 (19%) |
| +0.1 mM imazapyr | 0.172 (29%) | 0.038 (5%) |

[a] Control samples were acidified prior to the addition of enzyme. All values are the means of at least two separate determinations. [b] Leucine and valine were each present at 10 mM final concentrations in the assay medium. [c] Numbers in parentheses represent the activities as a percent of the respective no addition values.

Embryo-derived maize cell suspension culture ALS has a native $M_r$ of 440,000 (12) while maize kernel PDC is a very large protein that elutes near the void volume upon gel chromatography using Sepharose CL-6B (which has a fractionation range of 10,000–4,000,000) (7). Gel filtration was used therefore to separate embryo PDC from ALS. Upon gel filtration of maize kernel embryo extracts on a Superose 6 column (fractionation range 5000–5,000,000), PDC activity eluted as a single peak immediately after the void volume (Fig. 1A), as found previously (7). ALS assays of the fractions showed two peaks of creatine and $\alpha$-naphthol dependent color formation, one which co-eluted with PDC and was insensitive to inhibition by 10 mM leucine + valine and one which eluted much later, and whose color formation was dramatically reduced by the presence of 10 mM leucine + valine. In contrast, when extracts of endosperm (which do not contain PDC) were subjected to gel filtration under the same conditions, ALS assays revealed only one peak of activity which eluted in the same fractions as the later eluting, leucine + valine-sensitive embryo ALS activity (Fig. 1B).

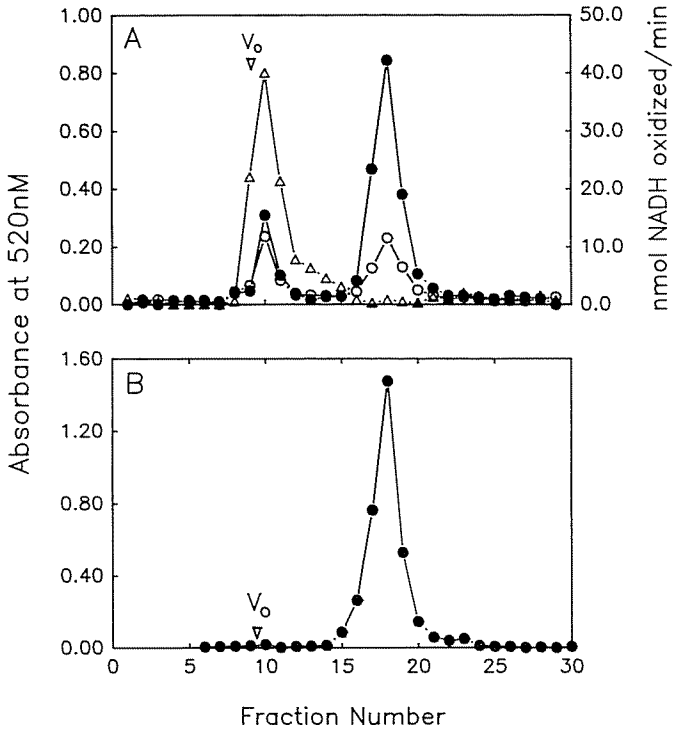


FIG. 1. Gel filtration of maize embryo (A) and endosperm (B) homogenates. Control ALS assays were acidified prior to the addition of enzyme. (●), Absorbance at 520 nm of α-naphthol and creatine dependent color formed in ALS assays of fractions; (○), same as before, but with 10 mM each leucine and valine included in the ALS assay medium; (△), PDC activity.

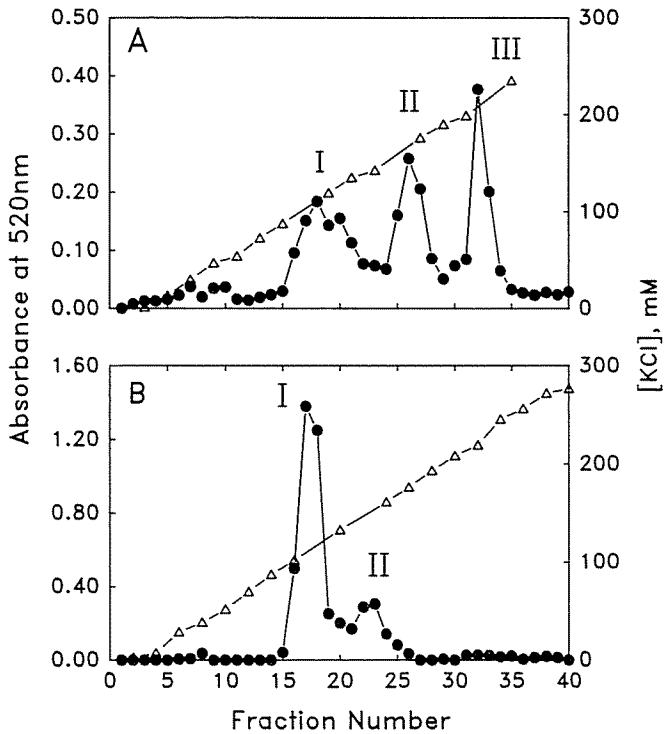


FIG. 2. Anion exchange chromatography of homogenates from maize embryo (A) and endosperm (B). Fifteen of 22 DAP endosperm or 60 of 30 DAP embryos were prepared as described in "Materials and Methods." The final sample volume was 3.5 ml in each case. Two and one-half ml samples were applied to the HR 10/10 column. Control ALS assays were acidified prior to the addition of enzyme. (●), Absorbance at 520 nm of α-naphthol and creatine-dependent color formed in ALS assays of fractions; (△), [KCl].

Embryo and endosperm extracts were also subjected to anion exchange chromatography. The profile of the embryo extract from the anion exchange chromatography contained three peaks of apparent ALS activity (Fig. 2A). Creatine and α-naphthol dependent color formation from ALS assays of peak pools I and II were inhibited by leucine + valine or imazapyr to the same degree as in ALS assays of crude endosperm extracts (Tables I and II). In contrast, the color formation from incubation of peak III pool aliquot in the ALS assay medium was inhibited only 25% by 10 mM leucine + valine and was completely insensitive to inhibition by 0.1 mM imazapyr (Table II). The peak III pool also exhibited high PDC activity which was insensitive to inhibition by leucine + valine and by imazapyr (Table II). Peak III was absent from anion exchange chromatographic profiles of endosperm extracts (Fig. 2B), consistent with PDC's localization in the embryo.

A surprising result of the ion exchange study which should be noted was the elution of multiple peaks of ALS activity upon chromatography of either the embryo or the endosperm extracts (peaks I and II, Fig. 2). Whether these peaks represent true ALS isozymes or are induced by extraction or chromatographic conditions will require further study.

The results of Figures 1 and 2 and Table II support the contention that PDC contributes to apparent ALS activity of crude embryo extracts under standard assay conditions, and that it is the insensitivity of embryo PDC to inhibition by leucine + valine and imazapyr which makes embryo ALS activity measured in this manner appear to be less sensitive to these inhibitors when compared to ALS activity from endosperm extracts. The data presented in Table II suggests that imazapyr can be used to correct measurements of ALS activities in crude extracts which contain PDC since only that portion of the color formed in the assay samples which is inhibited by 0.1 mM imazapyr is due to the action of ALS. Therefore, the absorbance of duplicate samples which contain 0.1 mM imazapyr could be subtracted from the absorbance of nonimazapyr containing samples to eliminate

Table II. *Acetolactate Synthase and Pyruvate Decarboxylase Activities of Selected Pooled Fractions from the Anion Exchange Chromatography of Maize Embryo Homogenates*

Two and one-half ml samples of fractions 17 + 18 (peak I pool), 25 + 26 (peak II pool), and 32 + 33 (peak III pool) from Figure 2A were desalted in ion exchange starting buffer (final volume 3.5 ml). ALS assays were performed using 175 μl of enzyme incubated at 37°C for 1 h. ALS assay absorbance values are ±1 SD. $n = 3$.

| | I | II | III |
|---|---|---|---|
| | Apparent ALS Activity[a] *absorbance at 520 nm* | | |
| No additions | 0.121 ±0.018 | 0.134 ±0.015 | 0.133 ±0.013 |
| +10 mM leu + val | 0.024 ±0.004 (19.8%)[b] | 0.000 ±0.005 (0.0%) | 0.100 ±0.009 (75.2%) |
| +0.1 mM imazapyr | 0.007 ±0.006 (5.0%) | 0.004 ±0.004 (3.0%) | 0.126 0.01 (94.7%) |
| | PDC Activity *μmol NADH oxidized/min* | | |
| No additions | 1.5 | 1.6 | 26.6 |
| +10 mM leu + val | | | 23.3 (88%) |
| +0.1 mM imazapyr | | | 27.9 (105%) |

[a] Control assays were acidified prior to addition of enzyme. [b] Values in parentheses represent the activities as a percent of the respective no addition values.

that portion of the color formation which is due to PDC activity. It should be noted, however, that this method is only valid when the ALS assay is performed with saturating concentrations of substrates and cofactors. Under nonsaturating conditions, PDC may outcompete ALS for pyruvate or cofactors, leading to an overestimation of PDC contamination.

It is interesting to note that PDC activity is found in the roots as well as the seeds of maize (7). The presence of PDC in the root tissue may account for the apparent insensitivity of maize root ALS activity to imidazolinone inhibition when compared to maize shoot ALS activity (14). Interfering PDC could likewise account for the apparent differences in pea seed and leaf ALS activities with regard to leucine + valine sensitivity (10).

**Effect of Kernel Age on Endosperm and Embryo Acetolactate Synthase Activity.** The activity of ALS in freeze-dried maize endosperm and embryos as a function of kernel age is shown in Figure 3. Total ALS activity/unit endosperm increased rapidly during early kernel development to a peak of approximately 3 μmol acetoin/h at 25 DAP and then slowly declined throughout the remainder of development. Activity of ALS in the embryo increased throughout the first 40 d of kernel development, then remained constant through the remainder of the sampling period (Fig. 3). Maximum embryo ALS activity (at 40 DAP) was approximately one-tenth that of the maximum endosperm activity (25 DAP), and only at 50 DAP was embryo ALS more active than endosperm ALS, on a per unit tissue basis. The use of freshly harvested kernels or the addition of 1 mM phenylmethylsulfonyl fluoride, 0.5 M NaCl, or 0.5% (v/v) Triton X-100 in the extraction media did not alter the patterns of ALS activities found in endosperm or embryo extracts as a function of kernel development (data not shown).

The peak of endosperm ALS activity which occurs at 25 DAP precedes the bulk of total nitrogen accumulation (Fig. 3), similar to the patterns found for other enzymes of amino acid metabolism during endosperm development, including aspartate kinase (5), diaminopimelate decarboxylase (18), glutamine synthetase (4, 8, 11), glutamate synthase (11, 19), threonine deaminase, and dihydrodipicolinic acid synthase (MJ Muhitch, unpublished data). These patterns suggest a coordinated regulation of gene expression for the enzymes of nitrogen assimilation and amino acid biosynthesis during maize endosperm development. In contrast, embryo ALS activity, which is modest in comparison to the endosperm activity, parallels the increase in embryo total nitrogen content.

When ALS was measured using the standard assay, embryo ALS appears to become less sensitive to inhibition by leucine + valine during kernel development (Fig. 4A). This apparent change in the feedback regulation of embryo ALS activity with kernel age reflects the appearance of PDC activity, the developmental expression of which is delayed approximately 8 to 10 d compared to embryo ALS activity, but which reaches maximum activity at the same age as embryo ALS (not presented). When the absorbance of duplicate assay tubes containing imazapyr were subtracted from these ALS assay samples, the sensitivity of embryo ALS activity to inhibition by leucine + valine remained constant throughout kernel development (Fig. 4A). Leucine and valine sensitivity of endosperm ALS activity did not change during development when measured with either the standard assay or measuring only imazapyr-sensitive color development

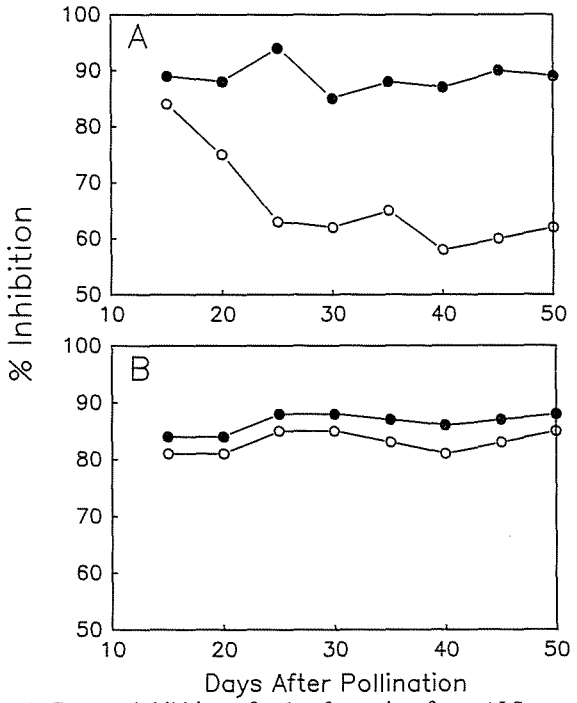


FIG. 4. Percent inhibition of color formation from ALS assays of various age embryos (A) and endosperm (B) by 10 mM each leucine and valine. (○), Standard ALS assay with control samples acidified prior to enzyme addition; (●), modified ALS assay using duplicate assay samples containing 0.1 mM imazapyr as controls. Each point represents the mean of assays of three separate tissue samples.

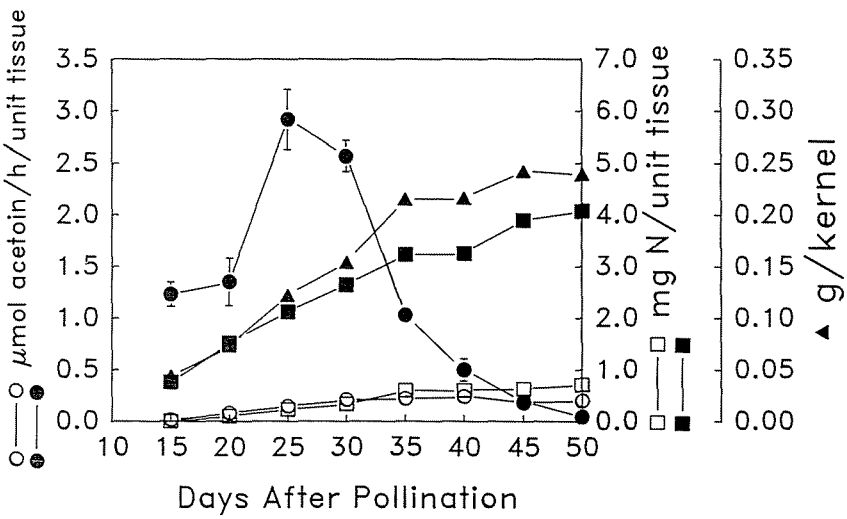


FIG. 3. ALS activities, total nitrogen contents and whole kernel dry weight, as a function of kernel age. All parameters are presented on a per unit (endosperm, embryo, or kernel) basis. Assay samples containing 0.1 mM imazapyr served as control assays for ALS activity measurements. ALS activities are the means from three independent samples for each kernel age. Error bars represent 1 SD. No error bars appear for standard deviations smaller than the symbols. (●), Endosperm ALS activity; (○), embryo ALS activity; (■), total endosperm nitrogen; (□), total embryo nitrogen; (▲), kernel dry weight.

(Fig. 4B). This result was as expected since maize endosperm does not contain PDC.

Few studies have addressed the potential biochemical interaction between the endosperm and the embryo with respect to nitrogen metabolism during maize seed development. In two reports (3, 4) the authors have concluded that the embryo plays a role in supplying amino acids for the endosperm during the later stages of kernel development. The pattern of changes in glutamine synthetase, glutamate dehydrogenase, alanine aminotransferase, and apartate aminotransferase activities in endosperm and embryo fractions as a function of kernel development (4) are similar to the results reported here for ALS. The author's claim that the capacity for glutamate and glutamine synthesis is greater in the embryo than it is in the endosperm is based on a comparison of glutamine synthetase and glutamate dehydrogenase activities expressed on a per gram fresh weight of endosperm or embryo. A similar conclusion could be drawn for ALS between 35 and 50 DAP. Expression of enzyme data on a fresh weight of tissue basis is, however, a rough measurement of enzyme concentration in the tissue and not a measure of the tissue's total catalytic potential in that the fresh weights of different tissues vary widely. Expressing enzyme activity on a per tissue (endosperm or embryo) basis is a more appropriate measure of total catalytic potential of a tissue. By this criteria, the endosperm has a much greater capacity for acetolactate synthesis (Fig.3 ), as well as for glutamate and glutamine synthesis and transamination (4), than does the embryo for all but the very last phase of kernel development.

Dierks-Ventling (3) has studied diaminopimelate decarboxylase activity (the terminal enzyme of the lysine biosynthesis pathway in plants) as a function of kernel development in the endosperm and embryo fractions of maize. Diaminopimelate decarboxylase activity declined sharply in the endosperm between 35 and 40 DAP while embryo activity rose rapidly during this same period to approximately one-half of the maximum endosperm activity. Based on these results and free amino acid pool data, Dierks-Ventling suggested that the embryo supplies lysine to the endosperm in the second half of kernel development (3). However, the role of the embryo in supplying lysine to the endosperm may be related to the rapid catabolism of this amino acid in the endosperm (2, 20). Certainly, further studies are necessary to more fully understand the nature of the metabolic interaction between the embryo and the endosperm during seed development. The results presented here on ALS are consistent with the embryo not playing a major role in supplying branched-chain amino acids to the endosperm during maize seed development.

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